A membrane-tethered transcription factor defines a branch of the heat stress response in *Arabidopsis thaliana*

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In plants, heat stress responses are controlled by heat stress transcription factors that are conserved among all eukaryotes and can be constitutively expressed or induced by heat. Heat-inducible transcription factors that are distinct from the "classical" heat stress transcription factors have also been reported to contribute to heat tolerance. Here, we show that *bZIP28*, a gene encoding a putative membrane-tethered transcription factor, is up-regulated in response to heat and that a *bZIP28* null mutant has a striking heat-sensitive phenotype. The heat-inducible expression of genes that encode BiP2, an endoplasmic reticulum (ER) chaperone, and HSP26.5-P, a small heat shock protein, is attenuated in the *bZIP28* null mutant. An estradiol-inducible bZIP28 transgene induces a variety of heat and ER stress-inducible genes. Moreover, heat stress appears to induce the proteolytic release of the predicted transcription factor domain of bZIP28 from the ER membrane, thereby causing its redistribution to the nucleus. These findings indicate that bZIP28 is an essential component of a membrane-tethered transcription factor–based signaling pathway that contributes to heat tolerance.

BiP2 | bZIP28 | endoplasmic reticulum | signal transduction | thermotolerance

As sessile organisms, plants must complete their life cycle in an ever-changing environment. To help cope with these fluctuating environmental conditions, plants have evolved particular responses for individual stresses and various combinations of stresses (1). The heat shock response involves the accumulation of molecular chaperones or heat shock proteins (HSPs) that stabilize partially unfolded proteins (2, 3). The induction of HSPs by heat stress is controlled by heat stress transcription factors (HSFs) that bind heat stress elements (5′-AGAAnnTTCT-3′) and are conserved among eukaryotes (2, 4). Plants are unusual in having 20 or more genes that encode HSFs, whereas other eukaryotes contain 1 to 3 of these genes. The expansion of HSF genes in plants is thought to result from a combination of diversity in activity and expression patterns of individual family members (2, 4). When temperatures are optimal, constitutively expressed HSFs are down-regulated by HSPs that bind and retain HSFs in the cytosol. During heat stress, these HSP-HSF complexes dissociate, allowing HSFs to redistribute to the nucleus and regulate gene expression (2, 5–7). The transcriptional activity of HSFs is also dependent on their phosphorylation state (7, 8). The heat stress response is attenuated by HSPs and the heat shock factor binding protein 1 (HSBP1), which binds to and inhibits HSFs (7).

Transcription factors that are induced by heat and are not related to these "classical" HSFs have recently been reported to contribute to heat tolerance in plants. DREB2A, an ethylene-responsive element-binding factor/Apetala 2-type transcription factor that contributes to drought and heat tolerance (9, 10), contributes to heat tolerance by inducing *HsfA3* expression (11, 12). Also, an *Arabidopsis* relative of the human nuclear transcription factor X-box binding 1 that contributes to salt and defense responses (13, 14) was recently shown to be heat inducible and to contribute to heat tolerance (15). These findings give evidence of cross-talk between heat stress and other stress signaling pathways.

Membrane-tethered transcription factors (MTTFs) are maintained in an inactive state by associating with membranes through one or more transmembrane domains (TMDs). In response to specific signals, an MTTF fragment that contains the transcription factor domain but lacks a TMD, is released from membranes by regulated intramembrane proteolysis (RIP), is redistributed to the nucleus, and regulates the expression of particular nuclear genes. MTTFs regulate diverse processes in prokaryotes and eukaryotes (16–20). Here, we show that bZIP28, which encodes a putative basic leucine zipper (bZIP) type MTTF, contributes to the up-regulation of heat-responsive genes and heat tolerance.

Results

The *bZIP28* gene is predicted to encode an MTTF with one (bZIP) domain, one TMD, and a site 1 protease (S1P) cleavage site and site 2 protease (S2P) cleavage site that contribute to RIP (refs. 16, 21, 22; supporting information (SI) Fig. S1). Genome expression data publicly available from AtGenExpress indicated that *bZIP28* is up-regulated in response to heat, a result that we confirmed (Fig. S2). These data lead us to postulate that bZIP28 might be an MTTF with a role in the heat shock response.

To test the membrane association and topology of AtbZIP28, we carried out cell fractionation experiments using *Arabidopsis* protoplasts that transiently expressed a fusion protein in which YFP was fused to the amino terminus of AtbZIP28 (YFP-bZIP28). YFP-bZIP28 accumulated in the pellet fraction, which contains cellular membranes as did the endoplasmic reticulum (ER) membrane marker GFP-calnexin (ref. 23; Fig. 1*A*). We did not observe an AtbZIP28 signal in either of the supernatant fractions, which contain soluble cytosolic and vacuolar proteins (site 1) and soluble microsomal proteins (site 2). An ER-targeted and -retained form of GFP (GFP-HDEL; ref. 24) was extracted in site 2 and pellet fractions, as expected for a soluble protein that localizes to the lumen of the ER (25). To determine the membrane topology of bZIP28, we digested fraction P with proteinase K. We observed that bZIP28, but not GFP-HDEL, was susceptible to proteinase K digestion in the absence of detergent (Fig. 1*B*). These data suggest that bZIP28 is oriented

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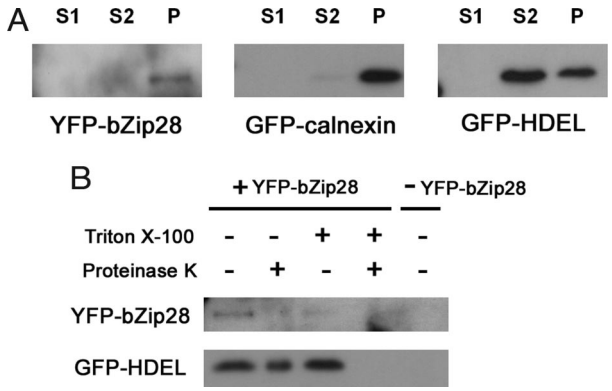

Fig. 1. Membrane topology analysis of bZIP28. (*A*) Cell fractionation. YFP-bZIP28, GFP-calnexin, and GFP-HDEL were transiently expressed in *Arabidopsis* protoplasts. Protoplasts were fractionated into supernatant 1, supernatant 2, and pellet fraction (P). Levels of YFP- and GFP-tagged proteins were monitored by immunoblotting with anti-GFP antibodies. (*B*) Membrane topology analysis of bZIP28. YFP-bZIP28, and GFP-HDEL protein levels were monitored as in *A* in *Arabidopsis* protoplasts that transiently express YFP-bZIP28 (+YFP-bZIP28) and in control cells that do not express YFP-bZIP28 (−YFP-bZIP28). Microsomes isolated from *Arabidopsis* protoplasts were treated with proteinase K, Triton X-100, or both.

with the amino-terminal domain in the cytosol, as would be expected for a type II or C-tail–anchored membrane protein, and that the bZIP domain is exposed to the cytosolic surface of cellular membranes, as would be expected for an MTTF.

To determine the subcellular localization of bZIP28, we expressed a YFP-bZIP28 protein in tobacco leaf epidermal cells. The YFP-bZIP28 signal emanated from the nuclear envelope, which is continuous with the ER (26), and, to a lesser extent, from the cytoplasmic strands of the ER (Fig. 2*A*) (compare distribution with the known ER marker GFP-HDEL; ref. 24; Fig. 2*C*), supporting our cell fractionation data (Fig. 1 *A* and *B*).

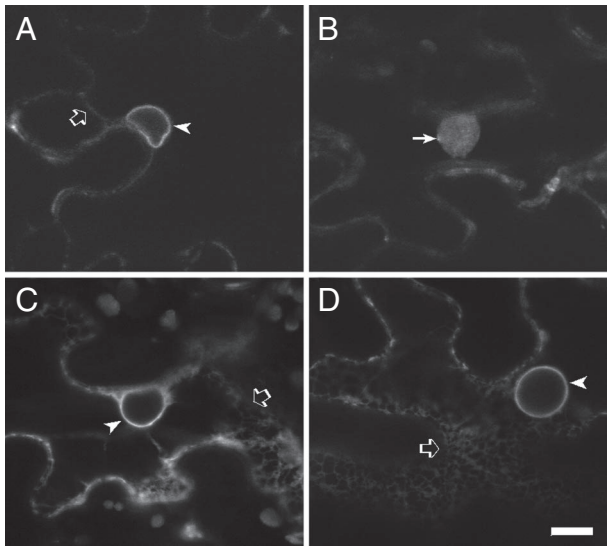

Fig. 2. Subcellular localization of YFP-bZIP28 before and after heat shock. Confocal laser scanning micrographs were prepared from tobacco leaf epidermal cells expressing YFP-bZIP28 imaged at 22°C (*A*) and immediately after heat shock at 42°C for 15 min (*B*). Confocal laser scanning micrographs were prepared from tobacco leaf epidermal cells expressing the known ER marker, GFP-HDEL, at 22°C (*C*) and immediately after heat shock at 42°C for 15 min (*D*). Arrow points to the nuclear envelope, and arrowheads point to the ER. (Scale bar, 5 μm.)

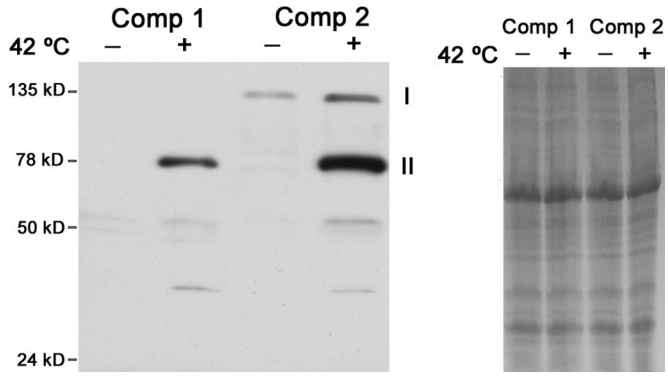

Fig. 3. Proteolysis and induction of YFP-bZIP28 during heat stress. Four-week-old *Arabidopsis* plants, as described in Fig. 2 and expressing YFP-bZIP28, were incubated at 22°C (−) or at 42°C (+) for 30 min. (*Left*) YFP-bZIP28 levels were monitored by immunoblotting with anti-GFP antibodies. The full-length YFP-bZIP28 fusion protein (Comp I) and the major proteolytic cleavage product (Comp II) are indicated. (*Right*) Coomassie brilliant blue staining of total proteins in SDS gels was used to test for equal loading.

Because *bZIP28* expression is induced by heat (Fig. S2), we hypothesized that heat stress might also trigger the movement of bZIP28 into the nucleoplasm. To test this idea, we monitored the subcellular localization of YFP-bZIP28 proteins before and after heat stress. YFP-bZIP28 fluorescence was observed mainly in the ER at 22°C (Fig. 2*A*), but when these cells were heat stressed at 42°C for 15 min, YFP-bZIP28 appeared in the nucleoplasm (Fig. 2*B*). Localization of YFP-bZIP28 in the nucleoplasm and the ER is most apparent from a three-dimensional reconstruction (Fig. S3). To test whether this apparent nucleoplasmic accumulation might be caused by a nonspecific disruption of the ER integrity, we also monitored the subcellular localization of GFP-HDEL under these same conditions. We found that GFP-HDEL did not move to the nucleus during a heat stress treatment in which YFP-bZIP28 was partially redistributed to the nucleoplasm (Fig. 2 *C* and *D*).

These data imply that the bZIP domain may move to the nucleus and regulate gene expression after being separated from the TMD by a mechanism that involves proteolytic cleavage, as has been reported for other MTTFs. To test this possibility, we monitored the size of the full-length YFP-bZIP28, which has a calculated mass of 100 kDa, in stable transgenic *Arabidopsis* plants by immunoblotting with anti-GFP antibodies before and after heat stress treatments. Heat stress elevated YFP-bZIP28 protein levels (Fig. 3), which is consistent with the observation that bZIP28 mRNA levels increased after heat treatments. Heat stress also induces a mobility shift in YFP-bZIP28 (Fig. 3), consistent with the cleavage of YFP-bZIP28 within the TMD. If cleavage occurs immediately before the TMD, a truncated YFP-bZIP28 fusion protein with a calculated mass of ≈63 kDa would be produced, which is approximately the size of the major species detected by immunoblotting in heat-treated plants. We suggest that the more rapidly migrating species in the heat-treated sample is derived from heat-induced cleavage of the YFP-bZIP28 fusion protein at the carboxy-terminus.

These data lead us to suggest that bZIP28 contributes to heat stress tolerance. To test this possibility, we induced heat stress in the *bZIP28* mutant that contains a T-DNA insertion in an exon of *bZIP28* (ref. 27; Fig. S4 *A* and *B*) The mutant exhibited a severe chlorotic phenotype after the heat stress treatment but was indistinguishable from WT under optimal growth conditions (Fig. 4). This mutant did not appear to have other visible phenotypes, such as morphological or pigmentation defects, when grown in optimal laboratory growth conditions for *Arabidopsis*. The chlorosis of WT plants was negligible compared with

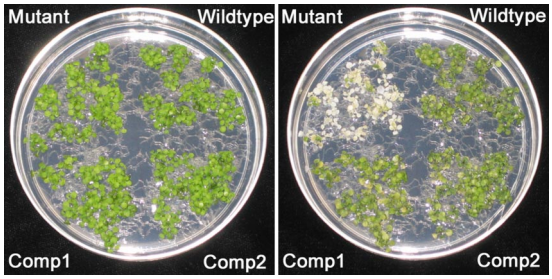

Fig. 4. Analysis of heat tolerance in a *bZIP28* mutant. Two-week-old WT plants, *bZIP28* mutants (Mutant) and *bZIP28* mutants containing a transgene in which a *bZIP28* promoter fragment drives the expression of YFP-bZIP28 (Comp 1 and Comp 2) were grown at 22°C (*Left*), incubated at 42°C for 2 h, and then grown at 22°C for 5 days (*Right*). This heat-sensitive phenotype was observed in two additional experiments (H.G., unpublished data).

the mutant and was similar to the *bZIP28* mutant that was stably transformed with a transgene expressing a fusion protein in which YFP was fused to the amino terminus of bZIP28 (YFP-bZIP28) (Fig. 4). A comprehensive RT-PCR analysis of the *bZIP28* T-DNA insertion allele indicates that bZIP28 mRNA does not accumulate in the bZIP28 mutant (Fig. S4*C*) and that partial RNAs can be transcribed from this allele upstream and downstream of the T-DNA insertion site (Fig. S4*D*). The observation that the heat-sensitive phenotype can be rescued by a transgene expressing the full-length bZIP28 fused to YFP indicates that the heat-sensitive phenotype results from a loss of bZIP28 function rather than from a gain of function like the transcription of aberrant mRNAs from the T-DNA insertion allele.

From these data, we hypothesized that bZIP28 is likely an MTTF that regulates the expression of heat-inducible genes. To identify candidate genes regulated by bZIP28, we searched publicly available data sets for genes that are up-regulated by heat and are coexpressed with *bZIP28*. Two genes identified during this search, At1g52560 and At5g42020, which encode the HSP26.5-P and the ER-localized chaperone BiP2, respectively, were up-regulated by heat less in the *bZIP28* mutant than in WT (Fig. 5*A*). The heat induction of *HSP17.4-CIII* (At1g54050) was indistinguishable in the *bZIP28* mutant compared with WT, as are 13 other genes that are heat induced and coexpressed with *bZIP28* (Fig. 5*A* and Table S1).

Our model predicts that a heat-induced signal releases bZIP28 from its membrane tether by triggering RIP and that the free bZIP domain contributes to the heat induction of genes such as *BiP2*. To test whether the bZIP domain of bZIP28 is sufficient to induce the *BiP2* gene, we used an estradiol-inducible promoter (28) to drive expression of a truncated version of bZIP28 that contained the bZIP domain but lacked the predicted TMD and the entire carboxy-terminus (i.e., bZIP28 Δ301–675). Estradiol treatments specifically induced expression of the bZIP domain and caused BiP2 mRNA to accumulate above the levels observed in the *bZIP28* mutant; accumulation of BiP2 mRNA levels correlated with the accumulation of bZIP28 mRNA levels (Fig. 5*B*). Elevated levels of *bZIP28 Δ301-675* expression were sufficient to induce nine other heat-inducible genes that are also induced by ER stress or encode ER-localized proteins with diverse functions (ref. 29; Fig. S5 and Tables S2 and S3). *HSP26.5-P* was not similarly induced by estradiol-inducible deletion construct (H.G., unpublished data), which indicates that although bZIP28 is necessary for optimal expression of *HSP26.5-P* (Fig. 5*A*), bZIP28 Δ301–675 alone is not sufficient for heat induction of *HSP26.5-P*. Although low levels of *BiP2* expression appeared to be induced by estradiol in the mutant, low levels of *BiP2* expression are induced by a variety of

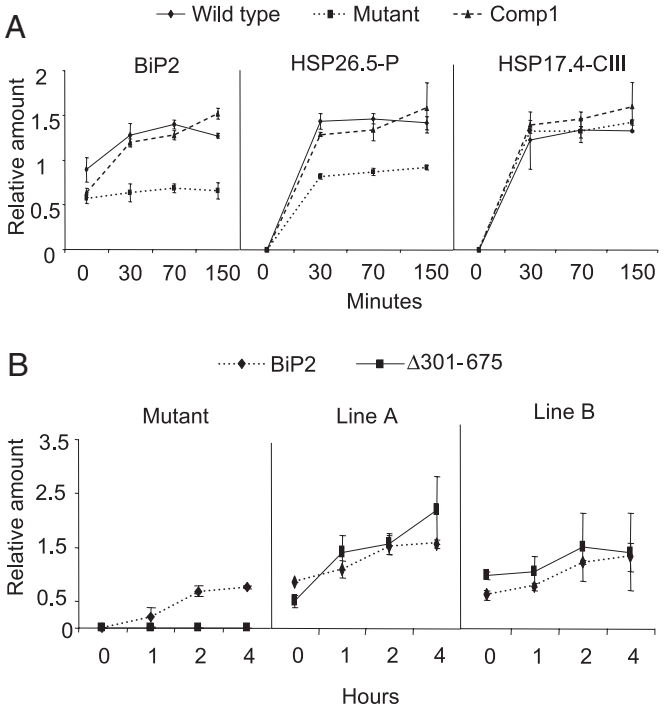

Fig. 5. Dependence of chaperone encoding genes on *bZIP28* for proper heat induction. (*A*) Analysis of heat-inducible gene expression in the *bZIP28* KO mutant. WT, the *bZIP28* mutant (Mutant), and the *bZIP28* mutant containing a transgene in which a *bZIP28* promoter fragment drives the expression of YFP-bZIP28 (Comp 1) were heat treated for the indicated periods of time. RNA was extracted from each plant, and the expression levels of *HSP26.5-P* (At1g52560), *BiP2* (At5g42020), *HSP17.4-CIII* (At1g54050), and *UBQ10* (At4g05320) were analyzed using semiquantitative RT-PCR. Two biological replicates were quantitated as described in *Materials and Methods*. Error bars represent SD. (*B*) Analysis of *BIP2* expression in transgenic plants that express an estradiol-inducible truncated bZIP28. The *bZIP28* mutant (Mutant) and two lines (Line A and Line B) that express an estradiol-inducible bZIP28 lacking amino acid residues 301–675 (Δ301–675) were treated with estradiol for the indicated periods of time. Analysis of expression levels for the indicated genes was as described in *A*.

manipulations similar to those used in this experiment (30). Low levels of induced expression were observed for *BiP2* regardless of whether seedlings were treated with estradiol (Fig. S6). These data indicate that low levels of *BiP2* expression are induced by experimental manipulations, as has been reported previously (30); that bZIP28 Δ301–675 is responsible for the remainder of this induction; and that estradiol does not affect *BiP2* expression independent of bZIP28 Δ301–675.

Discussion

Here, we show that bZIP28 is an ER-localized MTTF that contributes to heat tolerance. Heat appears to induce a signal that triggers the cleavage of bZIP28, followed by the redistribution of bZIP28 from the ER to the nucleoplasm. Once in the nucleoplasm, bZIP28 up-regulates heat-regulated genes. To our knowledge, such an MTTF-based signaling system has not been previously shown to contribute to heat tolerance in any system. Such a membrane-tethering mechanism suggests that bZIP28 may be tethered to the ER to respond to heat-induced ER stress. Consistent with this idea, unfolded protein stress within the ER was reported to trigger the redistribution of bZIP28 from the ER to the nucleus at optimal growth temperatures (22).

The redistribution of soluble transcription factors from the cytosol to the nucleus during heat stress is well established. HSFs are maintained in a dormant state at optimal temperatures by

forming a complex with HSPs in the cytosol. During heat stress, HSPs bind and stabilize misfolded proteins, thereby liberating HSFs, which redistribute to the nucleus and regulate gene expression (2, 3). Subsequent to the heat stress response, the activity of HSFs is attenuated and HSFs redistribute to the cytosol. In plants, HSBP1, HSP70, and HSP17.4-CII contribute to this down-regulation of HSF activity (31–34). Membrane tethering of bZIP28 and the formation of HSF-HSP complexes would appear to be distinct mechanisms that allow plants to respond rapidly to heat stress that is localized in the ER and cytosol, respectively. Proper attenuation of HSF activity appears essential in plants. For instance, null alleles of a gene that encodes HSBP1 in maize are embryo lethal, apparently attributable to an unattenuated heat stress response during embryogenesis (32). The tethering of bZIP28 to the ER suggests that bZIP28 is similar to HSFs in that the activity of bZIP28 is detrimental when temperatures are optimal. Indeed, driving bZIP28 expression by a strong and constitutive promoter severely inhibits plant growth at optimal temperatures (22).

In animal cells, MTTFs such as ATF6 and SREBP are retained in the ER by protein–protein interactions that can be disrupted by particular signals. Once these interactions are disrupted, the MTTF can be transported into the Golgi, where it is cleaved by RIP. This mechanism involves two distinct proteases known as S1P and S2P that cleave the MTTF between the transcription factor domain and the TMD (16, 19). In plants, bZIP17 and a plant protease that is related to S1P contribute to salt tolerance (21). Therefore, a similar signaling mechanism appears to be at least partially conserved between plants and animals. Such a signaling mechanism also seems likely for bZIP28, because bZIP28 appears to contain a canonical S1P cleavage site (21).

bZIP28 is related to three other MTTFs in *Arabidopsis*: bZIP17, bZIP49, and bZIP60. bZIP28 and bZIP60 regulate genes that are responsive to unfolded protein stress (22, 35), which is consistent with some redundancy among bZIP28 and its relatives. However, our analysis of a *bZIP28* mutant during a heat stress treatment not only indicates that bZIP28 is required for heat tolerance but that if other MTTFs contribute to this heat stress response, they are at most only partially redundant with bZIP28. Because bZIP17, but not bZIP28, was reported to promote salt tolerance (21, 22), at least this one bZIP28 relative is regulated by specific stresses and might affect distinct regulons, as has been suggested for MTTFs in animal cells (36). A system of ER-localized MTTFs that is regulated by overlapping and specific ER stresses may be beneficial for optimal regulation of ER-related processes in diverse environmental conditions.

Our findings not only suggest that bZIP28 is activated by a mechanism such as RIP during heat stress but indicate that *bZIP28* expression is induced during heat stress. These findings suggest that the bZIP28 activity may be required for an immediate heat stress response and that sustained bZIP28 activity may also contribute to heat tolerance. Our results also show that bZIP28 is required for the optimal expression of two heat-inducible genes that contribute to proper protein folding and thermotolerance in plants (2, 3). Additionally, high levels of the ER-localized BiP2 promote protein secretion in *Arabidopsis* (37). Therefore, a role for bZIP28 in the regulation of these genes is consistent with a role in protecting the ER from heat stress and promoting a proper unfolded protein response. Our initial gene expression analysis indicates that bZIP28 can induce several heat and ER stress-responsive genes that encode proteins of diverse functions when this factor is liberated from its ER tether. These initial results are consistent with a broad influence of the ER and bZIP28 on heat-responsive gene expression. Because the severe chlorotic phenotype of the *bZIP28* mutant developed over a period of 5 days after a brief heat stress treatment, the bZIP28-mediated transcriptome responses that are most important for heat tolerance may occur at any point during the heat stress treatment or during the subsequent recovery period at an optimal temperature.

Materials and Methods

Plant Materials and Growth Conditions. Plants were grown at 22°C under 12-h light and 12-h dark cycles. Light was provided by broad-spectrum fluorescent tube lamps at 120 μmol/m^{-2}s^{-1}. For heat stress experiments, 2-4 h after dawn, plants were transferred to a growth chamber with these same light conditions but with the temperature set at 42°C. The *bZIP28* T-DNA insertion mutant Salk_132285 (27) was obtained from the *Arabidopsis* Biological Resource Center (Ohio State University, Columbus, OH). The T-DNA insertion was verified by amplifying the insertion site using the following oligonucleotides: LBb1, 5′-GCGTGGACCGCTTGCTGCAACT-3′; 132285-LP, 5′-CACCATTAATTTCT-TAACCCAAGC-3′; and 132285-RP, 5′-GTTGCCTTAAAGCGACATTCTC-3′, in addition to Phusion DNA polymerase (New England BioLabs). The PCR product that contained the insertion site could be amplified using primers LBb1 and 132285-RP. Sequence data generated at the Research Technology Support Facility (Michigan State University, East Lansing, MI) with LBb1 confirmed that a T-DNA disrupted the first exon in Salk_132285.

Phenotypic analyses were conducted on 0.5 X Murashige-Skoog (MS) media (Sigma) containing 0.6% phytagar (Caisson Laboratories). The leaf tissue for immunoblotting was obtained from 4-week-old plants grown on soil.

Transient expression in *Arabidopsis* protoplasts was performed as previously described (38) and analyzed 12 h after transformation. Transient expression in tobacco was performed as previously described (39) and analyzed 2 days after infiltration.

Protoplast Fractionation. *Arabidopsis* protoplasts expressing YFP-bZIP28 were fractionated as previously described (40). Protoplasts were pelleted in 250 mM NaCl, gently resuspended in ice-cold extraction buffer (0.1 M Tris·HCl, (pH 7.8), 0.2 M NaCl, 1 mM EDTA), and incubated on ice for 10 min. The resuspended protoplasts were centrifuged for 15 min at 16,000 $\times$ *g* at 4°C in a microcentrifuge. The first supernatant, which contains cytosolic and vacuolar proteins, was removed and the pellet was resuspended by gentle sonication. The resuspended pellet was centrifuged for 15 min at 16,000 $\times$ *g* at 4°C in a microcentrifuge. The second supernatant, which contains soluble microsomes, was removed, and the pellet fraction, containing larger cellular membrane fragments and organelles, was resuspended in extraction buffer. Equal volumes of each fraction were analyzed by means of SDS/PAGE and immunoblotting using anti-GFP antibodies, which recognize GFP and YFP (Abcam).

Topology Analysis. Topology analysis was performed with a proteinase K protection assay, as previously described (40). Briefly, microsomes of *Arabidopsis* protoplasts expressing YFP-bZIP28 were prepared by osmotic shock in 100 mM Tris·HCl (pH 7.5), 12% sucrose, and 2 mM $CaCl_2$. The extracts were centrifuged at 1000 $\times$ *g* for 5 min, and the middle phase containing microsomes was removed. Microsomes were treated with 0.2 mg/ml proteinase K with or without 1% (vol/vol) Triton X-100 for 20 min on ice. Inactivation of the proteinase K was achieved by boiling for 10 min. One percent (vol/vol) Triton X-100 was added to samples that had not previously been treated with the detergent to release proteins from the membranes. Equal volumes of microsomes were analyzed by immunoblotting, as described in *Cell Fractionation*.

Bioinformatic Analysis. BLAST searches of the protein database were done at The *Arabidopsis* Information Resource (TAIR; http://arabidopsis.org/Blast/) and the National Center for Biotechnology Informaton (NCBI; http://www.ncbi.nlm.nih.gov/BLAST/). The prediction of the TMD was obtained from the ARAMEMNON database (41). N-glycosylation sites were predicted using the NetNglyc server (http://www.cbs.dtu.dk/services/NetNGlyc/). To identify genes that are coexpressed with *bZIP28*, we downloaded microarray data from AtGenExpress through TAIR (www.arabidopsis.org) and analyzed these data using Microsoft Excel. Genes that were expressed at least twofold more in heat stress conditions with a maximum value larger than 30 were chosen for further analysis. The list of coexpressed genes was narrowed further by comparing the response of these genes to *bZIP28* during other abiotic stress treatments, namely, cold, salt, osmotic, genotoxic, drought, oxidative, wounding, and UV stress. We chose genes that, like *bZIP28*, are induced more by heat than these other treatments. ER-localized genes were identified using the SUBA database (42).

Construction of *YFP* Fusion Genes. For transient expression experiments, a *YFP-bZIP28* fusion gene was constructed that was driven by the cauliflower

mosaic virus 35S promoter. For this gene fusion, the coding region of the *bZIP28* gene, including the intron, was amplified using 5′-ATCGACTAGTGAATCAACATCCGTGG-3′, 5′-TCAGGTGGCTACGAGATGGAGACC-3′, and Phusion DNA polymerase, digested with SpeI and inserted between the SpeI and StuI sites in a modified pCAMBIA vector with an *EYFP* gene and a *BAR* gene (CAMBIA). The resulting plasmid was sequenced with gene-specific oligonucleotides.

For expression in stable transgenic plants, a *YFP-bZIP28* fusion gene was constructed that was driven by the *bZIP28* promoter. For this gene fusion, all DNA fragments were amplified as described above, except that the 1 kb of the promoter region was amplified using 5′-GGATTCTAGAAGATGCCAGCAAACA-3′ and 5′-CATGCCATGGTCATCGTCGGAGGATTCG-3′ and digested with XbaI and NcoI; the YFP gene was amplified using 5′-GATGTCATGATGAGCAAGGGCGAGGAGC-3′ and 5′-CGGTTCTAGACAAGACCGGCAACAGGATTC-3′ and digested with BspHI and MluI; the coding region and a 454-bp downstream region of *bZIP28* were amplified using 5′-GACGACGCGTGAATCAACATCCGTGGTTGCTCC-3′ and 5′-GCTCGGTCACCCTGATAGTGGCCTGGCTTC-3′ and digested with MluI and BstEII. These three fragments were ligated into a derivative of the transformation vector pCambia3302 (CAMBIA) cut by XbaI and BstEII.

To express an estradiol-inducible truncated bZIP28 protein lacking the TMD and residues carboxy-terminal to the TMD, the *bZIP28* coding sequence was amplified as described above using 5′-ATTCCTCGAGATGGAATCAACATCCGTGGTTGC-3′ and 5′-ATCCACTAGTTCAGACAGGCTTAGGATTTAACTTAGG-3′, digested with XhoI and SpeI, and inserted into pER8 (28).

Microscopy. Fluorescence imaging was performed using a Zeiss Laser Scanning Confocal Microscope 510 META. A 488-nm line of an argon laser was used to excite GFP, and a 514-nm line was used to excite YFP. Emission filters of 505–550 nm and 530–600 nm were used for GFP and YFP, respectively, with appropriate main dichroics. Samples were viewed with an 63× oil-immersion objective. Postacquisition image handling was done with Zeiss AIM software and Paint Shop Pro (Corel, Ottawa, Canada).

RT-PCR Analysis. For the heat induction experiment, plants were grown for 2 weeks on 0.5× MS media, as described above. Mutant and WT plants were placed in a 42°C growth chamber for 0, 30, 70, and 150 min. For estradiol-mediated induction of the bZIP domain, plants were grown for 1 week on MS media, as described above, and subsequently placed in a tube containing 0.5 X MS liquid medium and 2 μM estradiol (Sigma Chemical Co.) for 1, 2, and 4 h. The plants were then harvested and frozen in liquid nitrogen. Total RNA was isolated by using an RNAeasy kit (Qiagen). To compare gene expression levels among the samples, equal amounts of total RNA were used for semiquantitative RT-PCR analysis with a SuperScript One-step RT-PCR kit (Invitrogen). The primers used for each gene were as follows: At3g10800, 5′-CAACATCCGTGGTTGCTCCTC-3′ and 5′-ACTAGCAACCTTCTTGAGCTTAC-3′; At1g54050, 5′-TCAGATATCCAGGTTACAGTGGAGG-3′ and 5′-GTCTTAGGTTTCGGCGGCTG-3′; At1g52560, 5′-CTCCCACCCTAAATGAGTTCTTTCC-3′ and 5′-CTCCGCCTTAATGTCCTCAACC-3′; At5g42020, 5′-AGGCTACGAAGTTAGGATCAGTTATTG-3′ and 5′-CTCTCAGGATTAACAGCCGCC-3′; and At4g05320, 5′-TCAATTCTCTCTACCGTGATCAAGATGCA-3′ and 5′-GGTGTCAGAACTCTCCACCTCAAGAGTA-3′. mRNA levels were quantitated using the Quantity One 1-D Analysis Software (Bio-Rad) and normalized to UBQ10, which had been quantitated using the same software.

For the comprehensive RT-PCR expression analysis of the bZIP28 T-DNA insertion allele (Fig. S4), RT-PCR was as described above, except that ACGAGGTACCGATGGAATCAACATCCGTGGTTGCTC and GAAATCTCCGTTCTCATCGTCGAG were used to monitor expression of this allele upstream of the insertion site and CAACGAAGGTGATGATGACGACG and AGACAAACCGCCAAAGCTTCC were used to monitor the expression of this allele downstream of the T-DNA insertion site.

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